THESIS

# THE SEROLOGICAL CLASSIFICATION OF STREPTOCOCCI

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THESIS

# THE SEROLOGICAL CLASSIFICATION OF STREPTOCOCCI

by

MARY MARGARET DONOVAN

(A.B., College of New Rochelle, 1947)

Submitted in partial fulfilment of the

requirements for the degree of

Master of Arts

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# I

## INTRODUCTION

Purpose:

The purpose in writing this paper is to compile in as complete a form as possible most of the available material on the subject "The Serological Classification of the Streptococci."

Historical Resume:

The earliest descriptions of the streptococcus were based on morphology and pathogenicity. In 1872, these organisms were demonstrated for the first time from a suppurative inflammation by Klebs. Pasteur described them as beads of chains seen in material from a patient dying of puerperal sepsis. Billroth noted the characteristic of streptococci of growing in chains and designated the organism as "chain coccus". In 1883, Fehleisen obtained streptococci in its first pure culture from a patient who had erysipelas. He produced this disease in a person suffering from sarcoma by the injection of a culture of what he called Streptococcus erysipelatis. The word streptococcus was first used in the generic sense by Rosenbach in 1884. He called strains isolated from acute abcesses, Streptococci pyogenes. Klein and others isolated streptococci from the throat of scarlet fever patients and called them Streptococci scarlatinae.

Early workers considered that strains of streptococci isolated from particular diseases were different species. This theory, however, was forsaken in 1886 when Fraenkel identified streptococci from puerperal sepsis with those isolated from erysipelas. In 1888, the streptococcus

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Early workers considered that strains of streptococci isolated from particular diseases were different species. This theory, however, was forsaken in 1890 when Prudden identified streptococci from puerperal sepsis with those isolated from erysipelas. In 1889, the streptococcus

was isolated from lesions of strangles in a horse by Schultz.

The effect of the streptococcus on blood was first described by Schottmueller in 1903. Mandelbaum, in 1907, stated that Streptococcus saprophyticus did not produce any visible effect on red blood cells. The three strains of streptococci yielding various effects on blood were classified in 1919 by Brown.[2] This was the basis of the first successful classification of streptococci.

The toxin production and activity of streptococci was studied by many, including McLeod,[34] Todd,[41] and Neill and Mallory.[35] The carbohydrate-splitting enzymes of this organism were also investigated for classification purposes. Among other methods studied in seeking for a complete and orderly classification were: (1) fermentation reactions and, (2) determination of bio-chemical and cultural characteristics including pH values necessary for growth and viability. These methods were not too successful because of the overlapping results which were usually difficult to interpret.

The possibility of a "Serological Classification" has always intrigued many investigators. The first attempts in this method made use of the agglutination reaction and agglutinin absorption. Many difficulties were encountered and around 1914 there was considerable controversy as to the value and interpretation of those results; whether concerned with the determination of serologically distinct type or with the differentiation of human and animal strains.[5] In 1918, the fact that specific serological types existed among hemolytic streptococci pathogenic for man was ascertained by a study of epidemic strains in the Army.[3]

A few years later, Hitchcock[12,13] discovered that strains of Streptococcus hemolyticus regardless of the human disease from which they were isolated all possessed a common serologically active polysaccharide. Heidelberger and Avery[10,11] had recently shown that the type-specificity of the pneumococcus was due to a bacterial polysaccharide, and it was hoped that this same principle would hold true for other bacteria. In the case of the streptococcus, S. viridans followed this rule but S. hemolyticus was the first exception.

Griffith[6,7,8,9] in England did a great deal of work in classifying the types of streptococci found in certain diseases including scarlet fever using the slide-agglutination method. Dr. Rebecca C. Lancefield of the Rockefeller Institute for Medical Research in New York City has successfully set up an antigenic classification of streptococci in groups and types. The results of her work have received wide recognition and are commonly used in diagnosing and treating streptococcal infection. It is this classification that we are concerned with in this paper.

Morphological and Cultural Characteristics:

Streptococci are Gram positive cocci occuring single, occasionally in pairs, and in chains. They are nonmotile, non-spore-forming and some forms have a capsule. They grow best on media to which some enrichment has been added. Their colonies are small, slightly dome-shaped and may be rough, smooth, or mucoid. They are usually grown on blood agar plates and have been classified according to their action on these plates into the following groups:

1. Gamma or Anhemolytic Streptococcus. This group does not produce

any visible effect, i.e. there is absence of hemolysis and methemoglobin on blood agar plates. Among the organisms found in this group are S. saprophyticus, S. cardioarthritis and S. fecalis. These are usually not or are only slightly pathogenic for man.

2. Alpha or Streptococcus Viridans. The organisms in this group produce a green zone surrounding the colonies and, therefore, are sometimes referred to as "Green Streptococcus". These are usually non-pathogenic organisms and are often found in the throats of normal individuals. They are sometimes found in low-grade infections. Among the members of this group are: S. salivarus, S. mitis, S. bovis, and S. equinus.
3. Beta-Hemolytic Streptococci or True Hemolytic Streptococci. These organisms which produce a clear zone of hemolysis surrounding the colonies are highly pathogenic for man. Included in this group are S. hemolyticus and S. pyogenes.

Streptococci may also be divided into aërobic and anaërobic groups. Most of them are aërobic, but there are a few pathogenic strains which grow best under anaërobic conditions.

Bio-Chemical Variations:

The streptococcus is not soluble in bile and usually does not ferment inulin. Acid but no gas may be produced in lactose, maltose, dextrose and sucrose. Gelatin is not liquefied. Hydrogen sulfide and indole are not produced.

Toxicity:

Beta-hemolytic Streptococci produce five toxic substances:

1. Streptolysin. This is a cellular component and was first used for classification purposes. It causes hemolysis of red blood cells and may be divided into two sub-groups:
   (a). Oxygen sensitive type (Streptolysin S). This toxin may be a reducing agent such as cystine.
   (b). Oxygen stable type (Streptolysin O). This toxin is destroyed by heat and by coming in contact with acid. It is not reactivated, however.
2. Streptoleukocidin. This toxin kills white blood cells and inhibits phagocytosis.
3. Erythrogenic toxin. This factor causes a rash with subsequent peeling in scarlet fever.
4. Streptokinase. This is a heat stable toxin. It acts like the Staphylococcus digesting fibrin once a clot has been formed.
5. Spreading factor (Duran-Reynal). This is a substance elaborated by the bacterium which facilitates the invasion of tissues by the organism.

Diseases Caused by Streptococci:

Streptococci differ from other organisms such as staphylococci in more general pathogenicity producing a varied assortment of diseases. The main infections caused by streptococci (beta-hemolytic streptococci) are: otitis media, a very serious throat which may be accompanied by a rash (scarlet fever), tonsillitis, Ludwig's angina, pneumonia (usually a secondary

infection), meningitis, puerperal sepsis, erysipelas, mastitis, acute-bacterial-endocarditis, arthritis and septicemia.

Streptococci of the green producing or viridans class are organisms of very low pathogenicity and are found in dental infections (in this case pathogenicity is uncertain), occasionally in suppurative lesions, and are invaders in sub-acute-bacterial-endocarditis.

## II.

## THE SEROLOGICAL CLASSIFICATION OF STREPTOCOCCI.

In the pneumococci, specificity is determined by a type-specific carbohydrate. Their nonspecific reactions are due to species-specific proteins. Hitchcock [12,13] found that a crude antiformin "residue antigen" yielded by most all of the hemolytic streptococci reacted with all of the antibacterial sera which he prepared against the hemolytic streptococci but did not react with sera prepared against the "green streptococci". Hirsch called the precipitating substance which he found in extracts of hemolytic streptococci the soluble specific substance.

All partially purified antiformin extracts and extracts from which the majority of the protein had been removed precipitated all antibacterial sera prepared against different strains of hemolytic streptococci. The precipitates formed were similar to those obtained with the type-specific carbohydrates in other species of bacteria. It was impossible, however, to separate type-specific fractions from these extracts. There was no trace

of a type-specific fraction in the methyl and ethyl alcoholic extracts of whole bacteria which contained the species-specific substance.

A type-specific fraction was found in extracts made by a modification of the Forges' method for removal of the capsule of the bacteria. The strains used were old and had been previously grouped by agglutination and precipitation. They were preserved by dessication while in a frozen state. These cultures had the same virulence, cultural and serological characteristics as they had at the time of dessication.

Methods of Preparation of Bacterial Extracts:

Bacteria were centrifuged from eighteen hour plain broth cultures. They were suspended in a 0.85 per cent solution of NaCl to which sufficient N/1 HCl was added to make a final concentration of N/20 HCl. Usually, the sediment from one liter of broth culture was extracted in 10 ml to 15 ml volume. The suspension was placed in a Pyrex centrifuge tube and was immersed in boiling water for fifteen minutes with occasional stirring. It was then cooled and centrifuged. The supernate was removed and neutralized with N/1 NaOH. The precipitate which appeared on neutralization was thrown down in the centrifuge, and the water-clear, slightly yellowish supernate was used as an antigen for precipitative tests. A more potent antigen was prepared by extracting in smaller volumes or by concentrating the dilute extract in the following manner. Crystals of sodium acetate (10 g / L extract) were added to facilitate the subsequent precipitation with three or four volumes of 95 per cent alcohol. After standing overnight in the icebox, the alcoholic precipitate was thrown down and redissolved in salt solution. The greater part of the alcoholic precipitate was insoluble, but precipitative tests showed very little loss of active material occasioned by discarding the insoluble part. The alcoholic precipitation was repeated and the antigen was concentrated to the desired volume by this means.[17]

Preparation of the Anti-Bacterial Sera:

Rabbits were inoculated with increasing doses of heat-killed broth cultures followed by living organisms. After six to eight weeks of immunization, the sera of such animals contained agglutinins and precipitins and, in some instances, protective antibodies for the homologous strains. Precipitens for antigens from strains of unlike types were also present in most of these sera.[17]

Results of the Precipitative Tests:

The results of the precipitative tests with unabsorbed antibacterial serum from a rabbit immunized with Strain S60, Type S60 showed the formation of heavy flocculent precipitates during the first incubation when the antigens of the homologous type were mixed with the serum.

The serum was precipitated slowly by heterologous antigens; no visible precipitate forming usually during the two hour period in the water baths. After standing overnight in the icebox, disc-like precipitates were formed. Once in awhile, non-type-specific precipitates appeared early and showed the flocculent characteristics of the homologous antigens.

The titer of the type-specific antibody tended to become lower after repeated absorption with the heterologous hemolytic streptococci. In this experiment nineteen strains were tested; ten yielded type-specific antigens, six gave non-type-specific antigens, and three did not produce any type specific antigens.[17] This may have been due to long cultivation or to unknown factors.

The results obtained from the precipitative test with absorbed serum are in agreement with those based on the agglutination and protection tests; and, therefore, can be used in classifying Streptococcus hemolyticus. However, it would be necessary to remove either the non-type-specific substances from the antigen or the non-type-specific antibodies from the serum, in order to obtain reliable type-specific precipitative reactions.

Group-Specific Substance "C".

The "C" substance parallels the type-specific carbohydrates of the pneumococcus in that it forms a typical disc-like precipitate with antibacterial sera. However, it differs from the polysaccharide of the pneumococcus and other bacteria by forming precipitates with antibacterial sera made from hemolytic streptococci of all types and therefore in being group-specific. The "C" substance is present in every streptococcal extract. It was not precipitated by the addition of three or four volumes of 95 per cent alcohol or by acetic acid. Therefore, the extracts used for the preparation of the type-specific protein substances were saved and employed in the isolation of "C".

The Antisera used were prepared in the manner mentioned above. In order to produce a serum which a high titer of "C" antibodies, intensive immunization was necessary.

Alcoholic Precipitation of the "C" Substance:

The solutions used for the precipitation of "C" were neutralized and concentrated to dryness, or to a small volume in vacuo at a temperature not exceeding 37 C. After concentration, distilled water was added to dissolve the salts, and the insoluble substances were discarded and three volumes of 95 per cent alcohol were added to remove some of the protein. After standing overnight in the icebox, the precipitate was thrown down in the centrifuge and discarded. The supernatant fluid was again concentrated in vacuo and was dialyzed in a colloidin sac against running water until most of the salts were removed.

The "C" substance was dialyzed through parchment membrane and also through colloidin sacs with the result that only an insignificant loss of active material was sustained in dialysis.

The test for colloids was negative so the solution was again concentrated in vacuo and sodium acetate in the proportion of 20 g/liter of solution together with twenty volumes of 95 per cent alcohol were added. This was left in the icebox overnight and the precipitate was removed by centrifugation. The supernatant fluid, concentrated and tested with potent

anti-"C" serum showed little "C" still present, and, hence it was discarded.

Lower concentrations of alcohol did not precipitate all of the "C" polysaccharide. The precipitate was freed of alcohol by evaporation and taken up in 16 ml. of distilled water; 0.6 ml of 1:1 HCl was added, and a heavy precipitate formed in the cold was removed by centrifyging.

The supernatant fluid was made strongly alkaline with NaOH and the gelatinous precipitate which formed was removed by centrifugation. Precipitative tests showed that neither the acid nor the alkaline precipitate contained much "C" - so they were discarded.

Another twenty volume alcoholic precipitate was made, followed by precipitations with acid and alkali as before; the acid and alkaline precipitates were discarded. This process was repeated four or five times until the addition of an acid or an alkali no longer produced a precipitate. The solution was then neutralized and two volumes of 95 per cent alcohol were added.

A gummy, deep yellow precipitate separated out, leaving only a small proportion of coloring matter in the supernatant fluid, from which, after concentration, a second two volume alcoholic precipitate removed still more gummy yellow material.

The two precipitates were combined, redissolved, and reprecipitated with two volumes of alcohol. Since the resulting precipitate did not contain much "C", it was discarded.

"C" polysaccharide was precipitated from the combined supernatant fluid by adding twenty volumes of alcohol; it was redissolved in distilled water and further purified by another removal of a gummy yellow substance by precipitation with two volumes of alcohol.

After the fourth addition of two volumes of alcohol, no more precipitate resulted. The pH was adjusted and absolute alcohol was added until a precipitate began to form. After standing overnight in the icebox, this precipitate was removed and was found to contain all of the "C".

After two such precipitations, the precipitate was dissolved in a small volume of distilled water made acid by the addition of a drop of 1:1 HCl and was dialyzed against running tap water for forty-eight hours and then against frequent changes of distilled water for forty-eight hours more. The solution was then concentrated <u>in vacuo</u> and thrown into a large volume of acetone. The resulting precipitate was dried and stored in a vacuum dessicator.[18]

The yield from this preparation was very small and therefore the information concerning the properties of C is meager. The final produce was a fluffy white powder which was colorless when dissolved in distilled water. All of the ordinary protein color tests gave negative results when applied to this solution. The Molisch reaction was positive. It only yielded 5 mg of nitrogen and resisted tryptic and peptic digestion. The nitrogen may

have been due to impurities. From the results of these tests, it is obvious that the "C" substance is not a protein but a carbohydrate. It may also be a hapten as described by Landsteiner, because the serum of rabbits immunized with extracts of Streptococcus hemolyticus never showed the smallest trace of a reaction with purified "C". The "C" substance is also capable of producing anaphylactic death in passively sensitized guinea pigs using amounts as small as 0.03 mg. However, the serum used must show a high titer of non-specific "C" precipitins.

With antibacterial sera prepared against both heterologous and homologous hemolytic streptococci, the "C" substance formed a precipitate. It did not, however, precipitate anti-nucleoprotein sera. The "C" substance is entirely non-type-specific in its serological reactions.

Type Specific Substance "M"

In extracts of hemolytic streptococci from which the non-type-specific antibodies were removed by using sera absorbed with the intact bacteria of heterologous hemolytic streptococci, it was possible to show type-specific antibodies with a precipitative reaction.

The best source of the type-specific substance called "M" was either N/20 or N/40 HCl extracts of bacteria.

Antibacterial sera was prepared by intravenous injection of killed and living cultures of the organisms. This was done in five series of four daily injections as follows:

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 1st | series | - | 4 | daily | injections | of | 1 ml | of | heat-killed cultures |
| 2nd | " | | " | " | " | | 2 ml | " | " " " |
| 3rd | " | | " | " | " | | 0.5 ml | " | " living " |
| 4th | " | | " | " | " | | 1 ml | " | " " " |
| 5th | " | | " | " | " | | 2 ml | " | " " " |

Between each series of injections, there was a resting period of three days. If the precipitin titer of the test bleedings was unsatisfactory, another series of three injections of 5 ml each of living culture was given. This was repeated until the titer was high enough.[18]

The "M" substance is obtained from the acid extract. This substance is precipitated with ordinary protein precipitants including picric acid. It gives a weak violet biuret test and a white precipitate which remained on heating and did not crystallize or become colored and was obtained with Millon's reagent. A faint color was produced on heating with nitric acid and this color deepened only slightly on the addition of ammonium hydroxide (xanthoproteic test). The Molisch test was not strongly positive. Using the micro-Kjeldah method, the total percentage of nitrogen equalled 14.6. There was only a small amount of amino nitrogen present. The acid equivalent was 552. The "M" antigen in neutral or acid solution can withstand autoclaving at fifteen pounds pressure for twenty minutes, but if in alkaline solution it is not as resistent. When digested with pepsin or trypsin, the ability of "M" to form precipitates with homologous antibacterial sera was completely gone. It did not stimulate antibody formation when injected into rabbits in saline solution. This was the only property which does not coincide with that of proteins. Therefore, "M" seemed to be a hapten rather than a true antigen. "M", also, produced typical anaphylactic shocks in passively sensitized guinea pigs.

Purified extracts of "M" reacted in high dilutions with homologous antibacterial sera, but did not form a precipitate with most of the heterologous antibacterial sera or with sera containing relatively large amounts of non-type-specific antibodies for the group-reactive nucleoprotein and species-specific probable carbohydrates.

The "M" substance is usually connected with virulence. It acts in a manner similar to that of the type-specific capsular polysaccharides in relation to pathogenic properties. It was later discovered that "M" antibodies would be produced in response to immunization with intact or dead streptococci or with solutions of "M" extracted from them. This appeared to be the cause of type-specific protection.

That the "M" substance in the living bacteria is readily accessible to the action of proteolytic enzymes without injuring the living cell's vital functions may be due to its possible location near the outer surface of the cell.

Non-Type-Specific Substance "P":

The non-type-specific substance "P" is even less specific than the non-type-specific carbohydrate. It is a true antigen stimulating the production of antibodies when injected into a rabbit. "P" shows marked but not absolute group reactions with nucleoproteins of other Gram positive cocci, including the non-hemolytic streptococci, the pneumococcus and staphlococcus. This substance is a nucleoprotein.

Non-Type Specific Substance "Y":

When "M" purified from certain strains of hemolytic streptococci not only gave type-specific reactions with most antisera, but also gave cross-reactions with some heterologous antibacterial sera which had not been absorbed with heterologous hemolytic streptococci. The fact that no reaction was obtained with high titer anti-"P" sera and also the intensity and range of these reactions indicated the presence of another non-type-specific substance. That it is a protein was shown by its digestion with trypsin

and with pepsin. From the evidence presented, it is a different protein from the non-type-specific nucleoprotein and is designated as "Y".

Antibody formation against all of these substances is induced by the intact bacterial cell, but only the nucleoprotein P has been found capable of stimulating antibody formation after the disruption of the cell body.

Group Differentiation of Hemolytic Streptococci:

Hemolytic streptococci are differentiated into several groups by use of the precipitin test. The first work on this classification done by Lancefield was based on 106 strains of Streptococcus hemolyticus isolated from a wide variety of sources including man, other animals, and dairy products. The precipitative reactions seemed to be dependent on the presence in the organisms of substances which are characteristic for groups, although not specific for the types within the groups.

It was previously thought that the carbohydrate -"C" substance was identical in all hemolytic streptococci. However, it has been shown not to occur in strains other than those isolated from humans. This apparent absence of "C" from hemolytic streptococci from lower animals has led many workers to determine whether similar substances might be found to differentiate the groups among other strains. This classification is based on the carbohydrate "C".

Methods:

1. Immune sera. Rabbits were immunized with formalized cultures as follows: The bacterial sediment from an eighteen hour broth culture was suspended in 1/20 volume of 0.85 per cent NaCl solution to which formalin was added in a final concentration of 0.2 per cent. After forty-eight hours in the icebox, these bacterial suspensions were sterile. Immediately before use they were diluted with physiological salt solution to the original volume of the culture. Daily intravenous injections of 1 ml were given for a week,

followed by a week of rest. Two to four series of injections were made. Although good antisera were often obtained after two series, four or more courses were sometimes required. On the fifth day after the last injection test bleedings were made and the sera of animals showing a good titer were collected and stored in the icebox.

All strains tried gave usable antisera by this method, although some were better antigens than others. After serial subcultures in 10 per cent type-specific immune serum, the resulting culture was relatively devoid of type-specific substances and proved the best antigen for inducing formation of anti-"C" precipitins specific for each group.

Antisera for strains of human origin were chiefly those which had been prepared previously by the method already described employing increasing doses of heat-hilled organisms followed by living cultures. The method noted above was satisfactory for these strains. With many of the strains it was impossible to use the injection of living cultures, since too great a loss of animals results.

Antisera were treated with extracts of both homologous and heterologous strains of the same group in order to make sure of the presence of the group anti-"C" precipitins. The type-specific antibody for a sub-group or type was often present in addition to the group anti-"C" precipitin used in this classification, and was sometimes the only antibody present. Since this classification is based on anti-"C" precipitins reaction, it was essential in testing antisera to employ an extract of a strain of heterologous type but homologous group, as measured by the anti-"C" reactions.

2. Extracts. Extracts were made by a method previously employed in preparing the type-specific substance, "M", of strains of human origin.

3. Precipitative Tests. In performing this test, increasing amounts of extract were placed in a series of tubes, usually 0.4, 0.1 and 0.025 ml constituted the serum. The volumes were made up to 0.4 ml with physiological salt solution, and a control tube with 0.4 ml of the same diluent was included. A constant volume of 0.2 ml of undiluted antiserum was layered in each tube and allowed to stand for ten to thirty minutes either at room temperature or in the water bath at 37 C. in order to observe ring formation. The tubes were then shaken and incubated for two hours at 37 C. in the water bath. Final readings were made after the tubes had stood overnight in the icebox. Sometimes a larger series of dilutions were employed, but no difference in result were obtained.

4. Final pH. The final pH attained in 1 per cent dextrose broth was determined colorimetrically. Readings were made at the end of four days incubation. Some of the first readings were made in duplicate with methyl red and brom-cresol green as indicators, but the later readings were all made with brom-cresol green since with this indicator the color changes were easier to distinguish.

5. Hydrolysis of Sodium Hippurate. This test was made according to the method of Ayer and Rupp. The cultures were grown four days in infusion broth containing 1 per cent sodium hippurate; then 1 ml amounts of the clear supernatant culture fluid were added 0.3, 0.4 and 0.5 ml respectively of ferric chloride containing concentrated hydrochloric acid in the proportion of 2.5 ml/l. Uninoculated sodium hippurate broth incubated for the same period was used as a control, and sufficient ferric chloride was used in this test to insure complete clearing of the uninoculated control. Care was taken to shake the tube thoroughly as soon as the ferric chloride was added. If the reaction was positive, a heavy precipitate of ferric benzoate was formed which was insoluble in the excess of ferric chloride. If the reaction was negative, the hippurate and protein precipitates formed at first were redissolved in the ferric chloride leaving a clear solution. Known positive and negative cultures were included as controls in every series tested.

6. Reduction of Methylene Blue Milk. The method of R. C. Avery was used for this test as follows:

A 1 per cent solution of methylene blue was added to sterile milk to make a final concentration of 1:5000. 0.1 ml of fresh 18 hour broth culture was inoculated into 5 ml of methylene Blue milk and into control tubes of milk without the dye. Readings were made after twenty-four hours incubation and at intervals up to a week.

7. Growth on Blood Agar Containing Bile. Rabbit blood agar plates containing 10 or 40 per cent ox bile were used to test the ability of these strains to grow in the presence of bile. Control plates of blood agar without the addition of bile were always inoculated at the same time. Twenty-four hour readings were essentially the same as observations made after three or four days incubation.

8. Fermentation Reactions. One per cent trehalose and sorbitol were added respectively to tubes of Hiss serum water with Androde's indicator. Cultures were observed at intervals, and final readings recorded at the end of a week's incubation. Where neither substance was fermented and in case of doubtful growth subcultures were made on blood agar plates to determine the presence of variable organisms. When tests with both substances were negative, a second set of tubes was inoculated.

9. Lysis with Streptococcal Bacteriophage. These tests were performed by adding 1 ml of fresh 18 hour broth culture (without disturbing the sedimented blood) to 5 ml of plain broth and incubating for from two to three hours. To 1 ml of such a culture 1 ml of bacteriophage was added. This bacteriophage was derived from that isolated by Clark and Clark from sewage. Either the Berkefeld filtrate of a lysed culture, or the unfiltered culture, crystal clear after lysis by the phage, was used in these experiments. The only difference observed in filtered or unfiltered reagents was that the filtered phage was somewhat less active. Controls of culture and broth were also incubated. The tubes were incubated at 37 C. for from one to eighteen hours, but lysis was usually complete in one hour if it occurred

at all. Overgrowth or resistant forms frequently were observed if the tests were incubated overnight.[21]

The results of the anti-"C" precipitative tests were the determining factors in the specific groupings. Closely related titers were obtained from precipitin tests performed with extracts of two representative strains from Group A agsinst antiserum of a third strain of Group A. These two strains produced no cross-reactions with antisera prepared against hemolytic streptococci of the other groups determined. These results existed within the other groups, also. The anti-"C" reaction seemed to be specific for Group A and it is possible that a similar substance causes the specific reactions in the other groups studied and the lack of cross-reactions among the various groups. The extracts were prepared to prevent the occurrence of protein fractions and the formation of their antibodies.

From the results obtained, which were strong enough to make definite distinctions between the groups, it was possible to show a positive correlation between the serological reactions and the cultural and biochemical reactions of the different groups.

Group A Streptococci:

Group A hemolytic streptococci are composed for the most part of strains isolated from human diseases. The twenty-three strains classified as Group A gave positive precipitative reactions against other members of this group, but gave no cross-reactions with antisera from members of other groups. Confirmation of these results were made by biochemical and cultural tests. The results of these tests were those

anticipated from strains of human origin.

All but one strain (which gave a pH of 4.8) had pH values ranging from 5.0 to 5.2. None of the strains hydrolyzed sodium hippurate or reduced methylene blue milk within twenty-four hours, although two strains gave evidence of reduction after three days incubation. This may have been due to the size of the inoculum. A few strains grew on 10 per cent bile blood agar and one even showed growth on 40 per cent bile blood agar. Treholose and sorbitol fermentation tests, which are used to distinguish between the human and animal strain of hemolytic streptococci gave positive reactions in the first and negative reactions in the second sugar. All were partially susceptible to the action of streptococcal bacteriophage but this reaction was not marked.[21]

Matt and Glossy Variants:

The type-specific protein "M" previously discussed is found in all the matt form colonies of Group A. It is present in very minute amounts or is not present at all in the glossy form colonies of this group. These colonies are variants. The organisms isolated from the matt type colonies are highly virulent, while those isolated from the glossy type are for the most part avirulent.

The glossy variant agglutinates with immune serum containing the homologous type-specific antibody and stimulates the production of type-specific antibodies effective against both variants when injected into animals. This antiserum produces reaction with solutions of the "M" substance. This antiserum does not give passive protection to animals. These variations gave rise to the investigation of the glossy forms and the finding of another type-specific substance in Group A, Type I matt and glossy variants.

Type-Specific Substance "T":

This substance causes the most important type-specific agglutination in all types of Group A hemolytic streptococci. The type-specific "T"

antibody is of great significance in the type-classification based on the agglutination reaction, even though this antibody lacks protective value.

"T" has been difficult to isolate from the bacterial cell, and, therefore, the knowledge concerning its chemical nature and immunological activities is limited.

The techniques used in the precipitin test and the methods of preparing antisera used by Dr. Lancefield were the same as those described earlier. The agglutination tests were practically the same as those mentioned before.

A twelve to sixteen hour broth culture, if the growth was sufficiently heavy, and diffuse were used without treatment. If unsatisfactory in either respect, the bacteria were separated from the culture medium by centrifugation and resuspended in fresh broth. A modification of the broth was used for growth and resuspension of these cultures.

No difficulty was experienced with suspensions of glossy variants, but some of the matt variants were prone to spontaneous agglutination. One helpful device was to grow these cultures at room temperature overnight and then concentrate them by centrifuging and resuspending the bacteria in the desired volume of fresh broth. Much the same effect was obtained by concentrating the early growth of very young cultures incubated at 37 C. Although no difference was observed in the results of agglutination with cultures prepared in these different ways, a culture grown for sixteen hours at 37C. was used whenever possible. The prepared culture was added in a volume of 0.5 ml to equal volumes of serum which had been diluted serially in broth. The control series with normal serum, diluted the same as the immune serum, were invariably included, and the experiment was discarded unless this series was negative.

Absorption experiments were performed with either living bacteria or from those killed by heating from five minutes to one hour at 56 C. Usually two parts of undiluted serum were mixed thoroughly with one part of packed bacteria and were incubated at 37 C for thirty minutes. The serum was removed after centrifugation at high speed and if living bacteria had been used for absorption, the serum was sterilized by heating at 56 C for one-half hour. Comparative tests showed no difference in results attributable to the use of living, as contrasted with heat-killed bacteria for absorption, but living bacteria were usually employed in the present experiments to avoid possible inactivation of antigens by heat.[26]

A series of absorption experiments was set up in order to determine if another type-specific substance (later designated "T") other than "M" was causing agglutination. Several strains of matt and glossy hemolytic

streptococci were used as absorbing agents. Absorption experiments with whole bacteria of homologous and heterologous matt and glossy variants were then performed.

Aliquot parts of serum from a rabbit immunized with Type I Matt culture were mixed with living bacteria centrifuged from sixteen hour broth cultures of the strains used. The mixtures together with a tube of untreated control serum were incubated in a water bath at 37 C for one-half hour, then centrifuged immediately. The supernatant serum was removed and put in a tightly stoppered tube, which was completely immersed in a water bath at 56 C for one-half hour in order to sterilize it. The streptococci were invariably killed by this procedure.

Dilutions of these sera were prepared and used for agglutination and precipitin reactions and passive protection tests in mice.[26]

In the absorption experiments, all agglutinins were removed. However, when the type-specific matt and glossy variants of a heterologous nature were used, the agglutinins were not removed. From this, it was concluded that the type-specific agglutinability and agglutinins of these strains were identical.

These tests indicated the existence of another type-specific substance "T", in both the matt and glossy variants, whereas, the "M" substance occurs only in the matt form. The matt and glossy variants of some types showed no difference in type-specific agglutination. In other types of the variants serum from which the "T" antibody had been removed by a glossy form, agglutinated the matt variant. This appeared to be due to the "M" anti-"M" system. The glossy variant still retains the type specificity of its parent organism regardless of the differences in agglutination between it and the matt variant.

It is impossible to state whether "M" and "T" occur in combination with each other or whether they are separate chemical entities. It is true, however, that separate antibodies to the two substances are produced.

Although there would be many advantages in using the glossy variant in preparing type-specific agglutinating antibodies, this is hampered by failure of the variant to appear in all strains. The glossy variants are readily influenced by the presence of several non-type specific agglutinins in their antibodies.

Several other strains of group A hemolytic streptococci were studies. In four of the strains, the "M" substance was present in the matt form and the "T" substance was present in both the matt and the glossy variants. In all cases, the "M" and "T" antigens were characteristic of their respective type and of no other type.

One strain studied contained its own Type III specific substances, "M" and "T" but also contained a "T" antigen characteristic of that of Type I. It does not contain the Type I "M" substance. This strain gave all the type-specific reaction of Type III and the agglutination reactions specific to Type I and depended on the Type I antigen. The "M" precipitin and protection reactions of Type I were not given.

Serological Types in Group A:

Griffith established many of the types of Group A hemolytic streptococci. He used the rapid method of slide-agglutination at room temperature.

A few drops of a dilution of a powerful immune serum and a streptococcal suspension were mixed together on a slide. This gave a useful preliminary indication of the type which was confirmed by an absorption test.[6]

He first defined four serological types of group A. He worked primarily with organisms isolated from scarlet fever and puerperal sepsis and found that many from both diseases belonged to the same types.[6] In 1927,[7]

he studied two hundred and twenty-two scarlatinal strains. Seventy per cent of these strains fell into the four recognized types. Continuing his work, he published the results of his studies in 1934,[9] in which twenty-seven types of hemolytic streptococci were described; twenty-three of these types were found to be members of Group A, three types belonged to Group C and one type to Group G.

This classification of the types of Group A hemolytic streptococci has been universally adopted.

Pathogenicity of Types of Group A:

There is no correlation between the type of the organism and the disease which it produces; i.e. one organism of a certain type may be the causative agent in several different diseases. Also, there is no relationship present between the severity of the disease and the type of organism which causes it. One specific type may cause a certain disease present in various degrees of severity in a single epidemic. Therefore, the clinical manifestations of Group A streptococcal infection are not dependent on the presence of the type-specific substances, "M" and "T" but upon other factors such as the physical resistance to the disease of the host and secondary invading organisms.

There are few infections in humans caused by Group A hemolytic streptococci that are fatal. In such cases, the organism appears to be a secondary invader such as in puerperal sepsis or endocarditis. There are very few fatal cases where the primary infection has been caused by a Group A hemolytic streptococci.

The treatment of streptococcal infections of the Group A strain is being hindered by the increasing numbers of these strains which have become resistant to drugs, especially the sulfa drugs.

Streptolysin was found to be a characteristic of Group A hemolytic streptococci. Both streptolysin S and O are present in most of these organisms. Certain strains of Group A hemolytic streptococci also form erythroginic toxins which cause rash in scarlet fever, streptokinase, hyaluronic acid (considered to be a capsular substance and formed by mucoid strains).

Group B Streptococci:

This group of hemolytic streptococci includes strains isolated from mastitis in cows and milk from normal cows. The basis of this classification was on the common precipitative reactions of the strains. The only cross-reaction between members of this group and antisera for other groups of the hemolytic streptococci were observed with extracts of two of these strains. These two strains gave cross-reactions with antisera of Groups C and D. The reactions with Group B antisera were much stronger than the reactions with the other groups. All the serological characteristics of these strains were correlated with their biochemical and cultural reactions.

They fermented dextrose broth to a final pH range of 4.4 to 4.6. They all hydralyzed sodium hippurate but did not reduce methylene blue milk. These strains grew actively on blood agar containing even 40 per cent bile. They were similar to strains of A in fermenting trehalose but not sorbitol. They were not susceptible to bacteriophage.[21]

In this group, there were three odd strains. Two were of human origin and one was from a rabbit. One of the strains was from the throat of a child with scarlet fever who always drank unpasteurized certified milk.

Type-Specific "S" Substance:

This substance is responsible for the type-differentiation of Group B hemolytic streptococci. The first clue to its identity was its formation of a typical disc-like precipitate characteristic of the union between the bacterial polysaccharide and its corresponding precipitins. Chemical tests performed gave the following results:

> The protein tests were all negative. The total nitrogen, which was probably due mostly to impurities, was 2 per cent. The Molisch reaction was very strong even in considerable dilution. It was not digested by trypsin and pepsin.[22]

Chemical tests run simultaneously on the group-specific substance "C" showed that these two substances "C" and "S" were not identical.

"S" is serologically distinct in each type and reacts only with antisera of the homologous type.

Serological Types in Group B:

Stableforth, in England, first established the existence of specific types in Group B. He found three serological types from bovine mastitis which were sharply defined by their agglutinative and precipitative reactions.

Twenty-one strains were subdivided into serologically specific types by the use of antisera containing the anti-"S" precipitin as the dominant immune body. All except three of the strains were defined serologically into the three distinct Types I, II, and III.

Specific reactions were given by extracts of the majority of the strains with homologous type antisera. Slight cross reactions were observed with immune sera of heterologous types. The three unusual strains seemed to contain minute amounts of the type-specific polysaccharides. The

type-specific substance of two of these strains was concentrated by precipitation with alcohol to a high enough titer to give precipitin reactions which placed them in Type II. The remaining strain after similar concentration and precipitation proved to belong to Type I.

All of the type differentiations were confirmed by cross-protection tests in mice.

The type-specific polysaccharide was important in the virulence of the organism as shown by the decrease in mouse virulence when virulent microorganisms were so treated that the type-specific polysaccharide was not elaborated by them. This substance, designated as "S", seems to have a decided influence on the antigenic properties of Group B hemolytic streptococci and in some cases on their pathogenicity.

Approximately fourteen main and subtypes among bovine strains and five more types including eighty-six human strains have been defined using the slide-agglutination method of Griffith.[6] Only one of the human strains fell into a bovine type. From this, it can be concluded that human carriers are not as a rule the source of infectors in bovine mastitis, but they must be considered in attempts to wipe out the disease.

One of the serological types of Group B hemolytic streptococci described previously as homologous on further investigation proved to contain two closely related types which have been designated as Type Ia and Type Ib. These were distinguished by reciprocal absorption experiments.

Homologous organisms absorbed all the antibodies from their respective antisera. Heterologous organisms, on the other hand, absorb only the antibodies responsible for the cross-reactions. These types absorbed the anti-

bodies responsible for the cross-reactions between the two unrelated types, whereas other types of Group B hemolytic streptococci do not. The two types elaborate polysaccharide which are chemically and serologically related.

A variant of Group B, Type I was found which gave the same antigenic and serological reactions as the original strain. However, the variant had lost the powers of producing pigment and hemolyzing blood. All of its other functions including its virulence were identical with those of the parent organism.

Pathogenicity in Relation to Human Infections:

Group B hemolytic streptococci have occasionally been the causative agent either as primary invaders or more usually as secondary invaders in several human diseases including puerperal sepsis, endocarditis, terminal pyelonephritis, meningitis and pneumonia. These infections occasionally prove to be fatal. It causes bovine mastitis and when given off in large numbers in the milk of a diseased cow may give rise to outbreaks of diseases such as scarlet fever. This occurs rarely, if ever.

Extra-cellular substances are not usually elaborated by Group B hemolytic streptococci.

Group C Streptococci:

This group contains hemolytic streptococci which are isolated from two sources.

1. Nonpathogenic strains or strains of low pathogenicity (S. equisimilis) of human origin which are culturally indistinguishable from the Group A hemolytic streptococci. These strains ferment trehalose but not sorbitol. They do not hydrolyze sodium hippurate.

2, Strains isolated from various animal sources such as cattle, guinea pigs, rabbits, and most frequently, horses. These strains (S. zooepidemicus) ferment sorbitol but not trehalose, but S. equi, found in horses, ferments neither.

The results of the various other chemical tests done are as follows:

The final pH range in dextrose broth was 4.6 to 4.9. This is intermediate between the pH range of Groups A and B. Sodium hippurate was not hydrolyzed and methylene blue milk was not reduced except in a few exceptions after forty-eight hours incubation. One strain grew on 40 per cent bile blood agar. The other strain grew more or less abundantly on blood agar with 10 per cent bile. The majority of the strains of hemolytic streptococci susceptible to the bacteriophage are found in this group. Only two of the strains included in this Group C were not subject to lysis in these experiments.[21]

Serological Types in Group C:

Griffith's[7] first differentiated serological types of Group C hemolytic streptococci. They were Types VII, XX, and XXI. These strains have large matt type colonies and are probably similar culturally to the Types $B_1$ and $B_2$ of animal origin.

There are several type-specific substances among Group C strains, but the study of these has not been extensively pursued. Dr. Lancefield,[21] however, isolated a type-specific substance from Type XXI of Group C which was digested by trypsin. Neither this substance nor any other similar substance was not isolated from mucoid strain of animal origin.

In the Group C strains of one specific type, which caused nine separate outbreaks of suppurative arthritis in lambs in East Anglia, England, a type specific substance was isolated.[14] This was a protein which was separated from the group specific polysaccharide "C". These produce methemoglobin and, therefore, are similar to the alpha Group C strains from

bovine mastitis.

In the mucoid strains of Group C a polysaccharide is an important factor in virulence, but is not associated with type-specificity.

Streptolysin O is elaborated by the matt colonies of Group C hemolytic streptococci. Streptokinase is elaborated by the very large matt colonies of the Group C organisms. Another extracellular product, hyaluronic acid, is produced by the mucoid strains of Group C.

There is a close relationship between Groups C and G because they possess a common protein antigen not encountered in other strains, such as those of Group A. This antigen produces confusing cross-reactions between certain members of these two groups. This cross-relationship is not connected in any respect with the group-specific carbohydrate "C". When these groups are freed of this protein, they are serologically distinct. Antisera may be prepared in which this protein is removed by absorption with appropriate matt colonies. This antiserum may also be obtained from rabbits which have been immunized with strains that do not contain this particular antigen.

Group D Streptococci:

The strains classified under Group D were isolated from human and animal feces, milk and cheese. They appear to be often non-pathogenic, although one of the strains, Streptococcus zymogenes, has often caused certain human infections. They are of great importance in industry because of their appearance in milk and of their actions in butter and cheese.

The results of the chemical tests performed on this group are as follows:

The final pH range was between 4.2 and 4.3. There was slight hydrolysis of sodium hippurate and the organisms grew readily in methylene blue milk and reduced it overnight. They grew luxuriantly on blood agar containing as much as 40 per cent bile. They were variable in their fermentation of trehalose and sorbitol although six of the eight strains exam- 21
ined fermented both. They were not susceptible to lysis by bacteriophage.

Serological Types in Group D:

Three distinct types of Group D have been identified. There seems to be a common antigen which these types possess and which determines their type specificity. This substance does not appear to be a carbohydrate, but it may possibly be a protein.

Non-hemolytic varieties of Group D have been well investigated. Among these strains are S. fecalis and S. liquiefaciens. These organisms not only react as members of Group D, but also seem to belong to the same serological types as the hemolytic members of this group. S. zymogenes is one of the Group D strains which is classified biochemically and serologically. There is a close relationship on an antigenic basis of hemolytic and non-hemolytic strains of Group D. Group D hemolytic streptococci differ in their ability to hemolyze red blood cells of various animals. S. lactis differs immunologically from S. fecalis, although they agree in their other biochemical reactions.

Pathogenicity of Group D:

The members of Group D hemolytic streptococci are often included among the "enterococci" because of their relationship to the intestinal contents. The strains of this group have been found in a few cases of endocarditis and Ludwig's angina. These cases have proved to be fatal. The Group D strains isolated from these infections have been of the non-

hemolytic variety.

Group D strains fail to respond to sulfonamide therapy.

Group E Streptococci:

The three strains used in the investigation of this group were originally isolated from certified milk. They were formerly considered as members of Group C. This was due to the slight cross-reactions with C antisera, the marked susceptibility to bacteriophage, and the very limited growth on blood agar plates with bile.

The results of the various chemical tests are:

The final pH range was from 4.6 to 4.8. Sodium hippurate was not hydrolyzed and methylene blue milk was not reduced. There was no growth in bile blood agar plates even when the concentration of bile was only 10 per cent. Trehalose and sorbitol were both fermented. There was rapid lysis by bacteriophage.[21]

The distribution of Group E strains has not been very definitely established. One distinct type of Group E has been defined.

Other Groups of Streptococci:

Among the other groups of the hemolytic streptococci which have been established are G, F, H, K, L, M and N.

Group G are usually non-pathogenic organisms. They are closely related to the strains of Group C. One distinct type has been defined in this group. These strains along with those of Groups G, F, H and K were isolated from the human nasopharynx. The Group G strains have been found in a few fatal cases of puerperal sepsis, agranulocytosis and endocarditis. They elaborate the extracellular components streptolysin and streptokinase.

Groups F, H, and K are non-pathogenic, although strains of Group F were isolated from one fatal case of pansinusitis and one fatal case of

pneumonia with empyema.[29] The etiological relationship of these groups has not been definitely established. However, one distinct type of Group K and two distinct types of Group F have been defined.

Groups L and M contain organisms which were isolated from dogs and were identified around 1940 by Fry.[29]

Group N Streptococci:

It was previously thought that S. lactis and S. fecalis differed in their serological specificity but were identical in their biochemical reactions. S. fecalis is a member of Lancefield's Group D and attempts were made to place S. lactis in this group as a S. fecalis variant, that is, as S. fecalis var. lactis. However, Shattock and Mattick[38] differentiated these strains both biochemically and serologically.

S. lactis fails to grow in broth at 45 C and does not grow in broth at pH 9.6. It does not survive heating at 60 C for thirty minutes. S. fecalis, on the other hand, does grow and survive under these conditions. These organisms are practically identical in their fermentation reactions.

Serologically, it was found that no extracts of S. lactis reacted with S. fecalis or with Group D sera; and no extracts of S. fecalis or Group D. organisms reacted with S. lactis sera. S. lactis sera do not react with extracts of any of the groups previously established by Lancefield. In all of the serological tests, Lancefield's technique was used.[17,18] Therefore, it was concluded that S. lactis was one member of a new group of streptococci and this group was designated as N.

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Pathogenicity of Group N:

S. lactis is the organism which is connected with the souring of milk. It has been found in contaminated dairy utensils, but does not appear in aseptically taken milk samples. No one has been able to isolate it from the mouths and throats of cows or from human and bovine feces or from the soil. It was suggested that the organism is
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of plant origin.

S. cremaris has also been placed in this new group. However, its specificity is still not clearly defined.

## SUMMARY

The gram positive streptococci have been classified by various investigators according to their morphological, biochemical and cultural characteristics, pathogenicity, toxin production and antigenic structure.

Antigenically, the classification is dependent upon the group-specific substance "C", the type-specific substances "M", "T", and "S" and the non-type specific substances "P" and "Y". The "C" substance seems to be a carbohydrate because considerably purified extracts of "C" resisted tryptic and peptic digestion and were negative for the ordinary protein tests. It forms precipitates with antibacterial sera prepared against heterologous and homologous hemolytic streptococci. Although the evidence is slight, it is thought that the "C" substance is a hapten as defined by Landsteiner.

The type-specific substance "M" appears to be a protein. It is precipitated by the usual protein precipitates. It contains fourteen per cent protein nitrogen, is completely destroyed by trypsin and pepsin digestion and is slowly destroyed by removal of the amino group when treated with nitrous acid. Purified extracts of "M" precipitate homologous antibacterial sera but do not react with heterologous antibacterial sera or with sera containing large quantities of the non-type-specific substance "P" or the group-specific substance "C". It is probably a hapten because attempts to immunize rabbits with "M" have failed to produce antibodies. This substance is usually connected with virulence and is found only in the matt variant of the hemolytic streptococci.

## SUMMARY

The "T" substance is also type-specific. It occurs in both the matt and glossy variants of the hemolytic streptococci. It is of great significance in the type classification based on the agglutination reaction, even though it lacks protective value. It has proved difficult to isolate the "T" substance from the bacterial cell and therefore the information we have concerning its chemical and serological nature is decidedly limited. It has proved impossible to state whether "M" and "T" occur in combination with each other or whether they are separate chemical entities. The type-specific substance "S" is responsible for the type differentiation of the Group B hemolytic streptococci. It is a carbohydrate and is chemically distinct from the group-specific substance "C".

The non-type-specific substance "P" is a nucleoprotein whereas the non-type-specific substance "Y" is a protein. The "P" substance is a true antigen and shows marked although not absolute group reactions with nucleoproteins of other gram positive cocci.

These substances were obtained from hydrochloric acid extracts of the bacteria and were concentrated by repeated alcoholic precipitation. The antibacterial sera was prepared by intravenous injections of killed and living cultures of the organisms given in five series of four daily injections.

Hemolytic streptococci are differentiated into several groups by the use of precipitative test. These reactions are dependent on the presence in the organisms of substances which are characteristic for groups, although not specific for types within the groups. Even though

the "C" substance appears only in the strains isolated from humans, it has been the basis of this classification.

Group A hemolytic streptococci are for the most part composed of strains isolated from human diseases. There are many types of Group A hemolytic streptococci. Griffith described many of these types using the rapid method of slide agglutination at room temperature. His method has been universally adopted. The clinical manifestations of the Group A hemolytic streptococcal infections are not dependent on the presence of the type-specific substances "M" and "T" but upon other factors such as the physical resistance to the disease of the host and secondary invading organisms. The treatment of these infections is continually being hindered by the increasing number of strains of Group A which have become resistant to drugs, especially the sulfa drugs. The Group A hemolytic streptococci forms many extra-cellular substances including streptolysin S and O, an erythrogenic factor, streptokinase, and hyaluronic acid.

Group B hemolytic streptococci include strains isolated from mastitis in cows and milk from normal cows. The classification of the group is based on the common precipitative reactions of the various strains. The type-specific substance "S" is responsible for the type differentiation of this group. These streptococci have occasionally been the causative agents either as primary invaders or more especially as secondary invaders in several human diseases. The strains of this group do not usually elaborate extra-cellular substances.

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Group C hemolytic streptococci are usually non-pathogenic or are of low pathogenicity. They are culturally indistinct from Group A strains and are isolated from various animal sources. Streptolysin O and streptokinase are elaborated by the matt colonies of this group, while their mucoid strains form hyaluronic acid. Groups C and G are closely related because they possess a common protein antigen which is not encountered in the other strains.

The streptococci classified under Group D were isolated from human and animal feces, milk and cheese. They seem to be usually non-pathogenic and are often included among the "enterococci" because of their relationship to the intestinal contents. They fail to respond to sulfonamide therapy.

Group E streptococci were originally isolated from certified milk. Their distribution has not been definitely established.

Among the other groups of the hemolytic streptococci established are G, F, H, K, L. M and N. They are usually non-pathogenic and have been isolated from various sources.

S. lactis is a member of Group N. It is the organism connected with the souring of milk. It has never been isolated from the mouths and throats of cows or from human and bovine feces or from the soil. It may be of plant origin. S. cremaris is also a member of this group, although its specificity is still not clearly defined.

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